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Cloning and localization of the lepidopteran protoxin gene of *Bacillus thuringiensis* subsp. *kurstaki*

(larval toxin/antigen/plasmid/chromosomal DNA)

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ABSTRACT **_Bacillus thuringiensis_ subsp. _kurstaki_ produces a proteinaceous crystalline inclusion that is toxic for lepidopteran larvae. There are several size classes of plasmids in this organism and the presence of one or more has been correlated with production of this protein, defined as a protoxin. DNA fragments of _B. thuringiensis_ subsp. _kurstaki_, obtained by _Eco_RI digestion, were cloned into the vector Charon 4A. Recombinant phage were screened immunologically for the production of protoxin. Cells infected with one phage, C4K6c, produced antigen that was the same size as the protoxin and was toxic to _Manduca sexta_ larvae. A 4.6-kilobase-pair (kbp) _Eco_RI fragment from C4K6c was subcloned into pBR328 and in both orientations in pHV33. Both _Escherichia coli_ and _Bacillus subtilis_ containing these recombinant plasmids produced antigen that crossreacted with antibody directed against the protoxin. The various sized plasmids of _B. thuringiensis_ were purified and only an _Eco_RI fragment from the 45-kbp plasmid hybridized to phage C4K6c. One of the pHV33 subclones, pSM36, hybridized to the same size _Eco_RI/_Hind_III restriction fragments from plasmid or chromosomal DNA. The cloned _Eco_RI fragment contained a 0.9-kbp _Pvu_ II fragment that was also present in chromosomal but not in plasmid digests. The original clone was therefore of chromosomal origin, although very similar or identical protoxin genes were present in both the 45-kbp plasmid and the chromosome. Several acrystalliferous nontoxic mutants have been isolated that lacked the 45-kbp plasmid and in some cases all plasmids. All of the mutants contained the chromosomal gene but did not produce protoxin antigen.**

Bacillus thuringiensis subsp. *kurstaki* synthesizes a proteinaceous parasporal crystal that is toxic to lepidopteran insects (1). The crystal protein is a protoxin (ref. 2; apparent M_r = 134,000) that is converted after ingestion by a susceptible insect to the toxic product, a 68,000-dalton polypeptide (3). Several reports indicate that plasmids are involved in the expression of the crystal protein (4–7). However, the lack of a well-developed genetic system in *B. thuringiensis* has precluded detailed analysis of the exact role of one or more of these plasmids in protoxin production.

To further study the structure of the insecticidal crystalline protein of *B. thuringiensis* and the regulation of synthesis, we have undertaken the cloning of the gene coding for this protein. In this paper, we describe the isolation of a recombinant bacteriophage, C4K6c, consisting of cloning vector Charon 4A and DNA from *B. thuringiensis*. Cells infected with this recombinant phage produced protoxin antigen having the same molecular weight as the protoxin. In addition, phage lysates were toxic to tobacco hornworm larvae. A 4.6-kilobase-pair (kbp) *Eco*RI DNA fragment from C4K6c was subcloned into chimeric plasmids propagated in *Escherichia coli* and *Bacillus subtilis*. These clones produced antigenic proteins and the *E. coli* subclone was toxic to hornworm larvae. The 4.6-kbp fragment hybridized primarily to a 45-kbp plasmid of *B. thuringiensis*, as well as to chromosomal DNA. The hybridization data were consistent with the cloning of a chromosomal DNA segment containing the protoxin gene.

MATERIALS AND METHODS

DNA Isolation. Total cellular DNA of *B. thuringiensis* subsp. *kurstaki* was isolated from late exponential phase cells grown in 1 liter of YEG medium [1% Difco yeast extract/0.1% dextrose/0.2% KH_2PO_4/0.3% $(NH_4)_2SO_4$, pH 7.3]. The cells were harvested by centrifugation and suspended in 50 ml of TES (50 mM Tris·HCl/50 mM NaCl/5 mM Na_2EDTA, pH 8.0) containing 500 mg of lysozyme, and the suspension was incubated at 37°C for 30 min. The cells were lysed by adding $NaDodSO_4$ to a final concentration of 1%. The lysate was extracted with TES-saturated phenol until the interface was clear. The lysate was then extracted once with chloroform/isoamyl alcohol (96:4) and treated with RNase A (50 μg/ml) for 1 hr at 37°C. The DNA was extracted once with TES-saturated phenol and once with chloroform and collected by ethanol precipitation.

Plasmid DNA was isolated from 1-liter cultures grown to late exponential phase in YEG medium, essentially by the procedure of Casse *et al.* (8). Charon 4A DNA was isolated from 1 liter of lysate that was cleared of debris by centrifuging at 4,000 × *g* for 10 min. The phage were then concentrated by precipitation at 4°C with 120 g of polyethylene glycol 6000 and 0.5 M NaCl per liter of lysate. The precipitate was collected by centrifuging for 20 min at 7,000 × *g*. The pellet was suspended in phage adsorption buffer (50 mM Tris·HCl/100 mM NaCl/10 mM $MgSO_4$, pH 7.4) and extracted with chloroform/isoamyl alcohol (96:4). The phage was then pelleted from the aqueous phase by centrifuging at 44,000 × *g* for 1.5 hr. The pellet was suspended in phage adsorption buffer containing DNase and RNase A at 10 μg/ml each, and the suspension was incubated at 37°C for 60 min.

The phage was lysed by adding EDTA to 0.05 M and $NaDodSO_4$ to 0.5% and incubating the mixture at 65°C for 15 min. The lysed phage was treated with proteinase K (100 μg/ml) for 2 hr at 50°C, and the mixture was then extracted twice with

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Abbreviations: kbp, kilobase pair(s); TES, 50 mM Tris·HCl/50 mM NaCl/5 mM Na_2EDTA, pH 8.0.

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chloroform and twice with ether. The DNA was precipitated with 2 vol of ethanol and collected by centrifugation.

Enzymes. Restriction endonucleases and T4 DNA ligase for subcloning were obtained from Bethesda Research Laboratories and used according to the manufacturer's instructions. T4 DNA ligase for Charon cloning was prepared from *E. coli* K1100 by treatment with NM 989 λ T4 (9). Ligation using T4 DNA ligase was performed as described by Maniatis *et al.* (10).

Preparation of Charon 4A Arms and *B. thuringiensis* *Eco*RI Fragments (10). Charon 4A DNA was completely digested and *B. thuringiensis* chromosomal and plasmid DNA was partially digested with various concentrations of *Eco*RI for 1 hr at 37°C. The digested DNAs were sedimented through 10–40% neutral sucrose gradients and portions of each fraction were analyzed by agarose gel electrophoresis. Fractions of the Charon 4A DNA containing the 19-kbp and the 14-kbp fragments but not the two small fragments were pooled. The *B. thuringiensis* fractions that contained large amounts of 10- to 25-kbp DNA were pooled, dialyzed against water, and concentrated by lyophilization. The fragments were then dissolved in 10 mM Tris·HCl/1 mM EDTA, pH 8.0.

Antibody Preparation. Rabbit antiprotoxin serum was prepared as reported (11). IgG was purified by polyethylene glycol 6000 precipitation followed by DEAE-Affi-Gel blue (Bio-Rad) chromatography using the procedure recommended by Bio-Rad. IgG was iodinated by the chloramine-T method (12).

Radioimmunoassay of Extracts from Cells Infected with Recombinant Phage or Plasmids. Approximately 100–300 phage were plated on X-Gal plates (L agar containing 5-bromo-4-chloro-3-indolyl β-D-galactoside at 40 μg/ml) and incubated overnight. Colorless plaques were plugged by using Pasteur pipettes and the plugs were placed in 16 × 100 mm culture tubes containing 1 ml of L broth and 10 μl of an overnight culture of *E. coli* K802. The tubes were then shaken until the cultures lysed (about 12 hr). Polyvinyl chloride microtiter plates (Dynatech MC-2000) were coated with rabbit anticrystal IgG [5 μg per well in 0.1 M $NaHCO_3$ (pH 9.2)]. The wells were then washed three times with wash buffer (0.15 M NaCl/0.05 M NaH_2PO_4, pH 7.8/0.1% bovine serum albumin/0.5% normal rabbit serum). Two hundred microliters of lysate from each recombinant plaque was placed in a well and incubated for 4 hr. Each well was then washed four times with wash buffer and 5 × 10^5 cpm of ^{125}I-labeled IgG was added to each well in 200 μl of wash buffer. The mixtures were incubated overnight at 4°C, then the wells were washed five times with wash buffer and separated, and the products were assayed in a Searle gamma counter. A standard curve was determined each day by adding solubilized *B. thuringiensis* crystal to a Charon 4A lysate and was linear to at least 200 ng of crystal protein/ml.

Extracts of *B. subtilis* JH641, carrying plasmid or the cloning vehicle pHV33 (13), were prepared from 50 ml of early stationary phase cells growing in a nutrient sporulation medium (14) containing chloramphenicol at 5 μg/ml. The cells were pelleted in a Sorvall SS-34 rotor at 2,000 rpm for 15 min, washed once with 10 ml of TES and suspended in 50 μl of TES containing 100 μg of lysozyme. After incubation at 37°C for 10 min, the suspensions were sonicated for 2 min with a Branson microtip and centrifuged as above. The supernatant was saved and the pellet was suspended in 30 μl of 8 M urea/1% $NaDodSO_4$/50 mM dithioerythritol/2 mM phenylmethylsulfonyl fluoride, pH 9.6, and incubated at 37°C for 45 min. After centrifugation, the supernatant was pooled with the original and portions of each were removed to determine protein content (15). Various amounts (4–30 μg of protein) were spotted on Whatman no. 1 filter paper discs and air dried. The filters were then incubated with 50 μl of *B. thuringiensis* antiprotoxin antibody in 3 ml of antibody buffer (10 mM Tris·HCl/0.9% NaCl/1% bovine serum albumin, pH 7.2) on a rocker platform for 1 hr at 27°C. The filters were washed four or five times with antibody buffer and then incubated as above with ^{125}I-labeled protein A (1–2 × 10^6 dpm) in 3 ml of antibody buffer. The filters were washed four or five times with 200-ml portions of 10 mM Tris·HCl/0.9% NaCl, pH 7.6, air dried, and exposed with intensifying screens to Kodak X-Omat film for 6 hr at −70°C.

Bioassay of Phage Lysates. Two hundred fifty milliliters of *E. coli* K802 was grown in L broth and infected with either Charon 4A or C4K6c and incubated at 37°C until lysis occurred. The lysates were adjusted to pH 12.0 by adding 2 M NaOH to solubilize any protoxin (2). After 60 min, the pH was adjusted to 8.0 with 2 M HCl and the lysate was dialyzed against 4 liters of 20 mM potassium phosphate (pH 8.0). After dialysis, the samples were concentrated to 5 ml on a rotary evaporator. One milliliter of the concentrated lysate was applied to each of 5 cups of diet as described (16) and a single neonate tobacco hornworm larva was placed in each cup. The larva were allowed to remain on the diet for 7 days.

Cloning and Hybridization. C4K6c DNA was subcloned into the *Eco*RI sites of pBR328 or the chimeric plasmid pHV33 (13). The latter was propagated in *E. coli* HB101 selecting for ampicillin resistance and in *B. subtilis* JH641 containing chloramphenicol at 5 μg/ml. Ampicillin-resistant colonies transformed with pBR328 recombinants were screened in minipreparations for a 4.6-kbp insert. Colonies of ampicillin-resistant *E. coli* transformed with pHV33 recombinants were screened with ^{32}P-labeled nick-translated *B. thuringiensis* plasmids (10, 17). Two recombinants, pSM36 and pSM37, contained 4.6-kbp *Eco*RI inserts and were transformed into *B. subtilis* JH641 protoplasts.

DNA was prepared as described above from various acrystalliferous plasmid-free strains or from purified plasmids (1, 4). DNA was digested with various restriction enzymes (according to protocols from Bethesda Research Laboratories) and fractionated on 0.8% agarose gels in 40 mM Tris·HCl/25 mM NaOAc/1 mM EDTA, pH 7.8, at 3 V/cm for 14–16 hr. The gels were stained with ethidium bromide at 1 μg/ml and photographed, and blots were prepared, hybridized, and washed as described by Southern (18). In some cases, the last wash with 45 mM NaCl/0.45 mM Na citrate, pH 7.0, was omitted so that homology with as much as 35% mismatch could be detected (19). Molecular weights of fragments were determined by preparing *Eco*RI-digested λ DNA, mixing with the other digest (after enzyme inactivation), and preparing Southern blots that were probed with either λ or one of the clones.

One-half to one microgram of λ or plasmid DNA was used for nick-translation (10, 17) with [^{32}P]dATP/[^{32}P]dGTP for the former or [^{32}P]dCTP for the latter by following the New England Nuclear kit instructions.

RESULTS

Construction of Charon 4A Recombinants and Isolation of Phage Containing the Protoxin Gene. DNA fragments resulting from partial *Eco*RI digestion of *B. thuringiensis* DNA were ligated to Charon 4A arms and packaged, and aliquots of the packaging mixture containing about 1,000 plaque-forming units were plated on *E. coli* K802 by using 5-bromo-4-chloro-3-indolyl β-D-galactoside-containing agar. A total of about 20,000 plaques were obtained; approximately 60% were colorless and presumably recombinants. Two milliliters of L broth was added to each plate and the top agar was scraped off, treated with chloroform for 30 min, and centrifuged for 20 min at 15,000 × *g* to remove the agar. The supernatants contained about 3 × 10^5

phage per ml. These phage were used as a library of *B. thuringiensis* DNA.

Assay of Clones Containing the Protoxin Gene. About 2,000 individual recombinant phage plaques were selected and grown in *E. coli* K802. The lysates were assayed for the presence of the protoxin by radioimmunoassay. Three clones showed activity significantly greater than background. One of these, designated C4K6c, was selected for further immunological and toxicity studies. On the basis of a standard curve using solubilized *B. thuringiensis* crystal protein, cells infected with C4K6c produced about 50 ng of immunologically crossreacting material per ml of lysate.

Phage lysates were prepared and applied to diet. A total of 15 tobacco hornworm larvae were exposed to three separate lysates prepared from C4K6c-infected cells (Table 1). The amount of protoxin antigen in C4K6c lysates was measured by radioimmunoassay and less than 2.5 μg per cup was required to kill 95% of the hornworm larvae. This quantity was less than 5 times the amount of pure parasporal crystal protein required to kill 95% of the larvae (Table 1). The larvae exposed to the Charon 4A lysate, as well as other control treatments (see Table 1), grew and developed as well as untreated larvae.

Hybridization of C4K6c DNA to *B. thuringiensis* Plasmids. In view of the large number and variety of plasmids that have been found in *B. thuringiensis* strains (4, 5), it was of interest to determine whether the DNA cloned in C4K6c originated from one or more of the plasmids. Purified fractions of all of the major classes of plasmids of this strain were digested with *Eco*RI, and Southern blots prepared and probed with nick-translated C4K6c DNA (Fig. 1). Charon C4K6c contained an insert that was cleaved to six fragments by *Eco*RI (lane A), with sizes, in decreasing order, of 4.6 kbp, 2.3 kbp, 2.1 kbp, 1.25 kbp, 1.05 kbp, and 0.6 kbp. Among the plasmid species analyzed, only the 45-kbp plasmid (lane C) showed strong hybridization to nick-translated C4K6c, in particular an *Eco*RI fragment similar to the 4.6-kbp fragment of Charon C4K6c (fragment 1 in lane A). There was weak hybridization to an *Eco*RI fragment of this same molecular weight in digests of the 70-kbp plasmid (lane B). It was difficult, however, to rule out a slight cross-contamination of this plasmid with the 45-kbp plasmid.

Subcloning of C4K6c into a Chimeric Plasmid. The presence of a large *Eco*RI fragment in the 45-kbp plasmid hybridizing to the clone suggested that this fragment contained the protoxin gene. An *Eco*RI digest of C4K6c was cloned, therefore, into the chimeric vector, pHV33, as well as into pBR328. Ampicillin-resistant transformants of *E. coli* HB101 and chloramphenicol-resistant transformants of *B. subtilis* JH641 were screened in minipreparations for large inserts or by colony hybridization to *B. thuringiensis* plasmids. Several transformants of both bacteria were found to contain a 4.6-kbp insert and both orientations were established by comparing *Pvu* II digests (Fig. 2). Protein antigen was produced in *B. subtilis* (Fig. 3) and in *E. coli* (unpublished results) by one of these clones, pSM36. The size of the antigen has not yet been determined.

Table 1. Insecticidal activity of Charon C4K6c-infected cells

Preparation	Toxic amount, μg
C4K6c lysate*	<2.5
Parasporal crystal†	0.5

Toxicity is expressed as the amount (μg) of protoxin required to kill 95% of larvae within 48 hr and was determined by the method of Schesser *et al.* (16), which uses neonate larvae of the tobacco hornworm *Manduca sexta* L.

* Antigen concentrations in λ recombinant phage lysates were determined by radioimmunoassay. Equivalent amounts of a lysate from Charon 4A-infected cells, intact *E. coli* K802, and bovine serum albumin had no toxic effect. The larvae developed to fifth instar within a 7-day period.

† Protein concentration measured by the method of Lowry *et al.* (15).

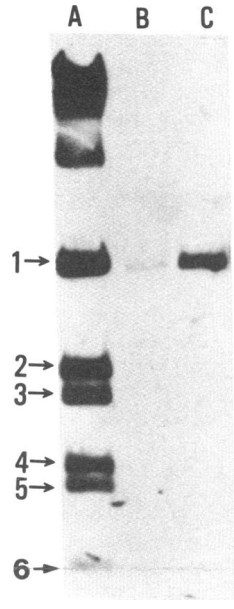

FIG. 1. Southern hybridization analysis of 45- and 70-kbp plasmids. Autoradiogram of ^{32}P-labeled C4K6c DNA hybridized as follows. Lane A: *Eco*RI digest of C4K6c; fragments derived from *B. thuringiensis* were 1, 4.6 kbp; 2, 2.3 kbp; 3, 2.1 kbp; 4, 1.3 kbp; 5, 1.05 kbp; 6, 0.6 kbp. The two unmarked bands are DNA from the cloning vector Charon 4A. Lane B: *Eco*RI digest of purified 70-kbp plasmid. Lane C: *Eco*RI digest of purified 45-kbp plasmid.

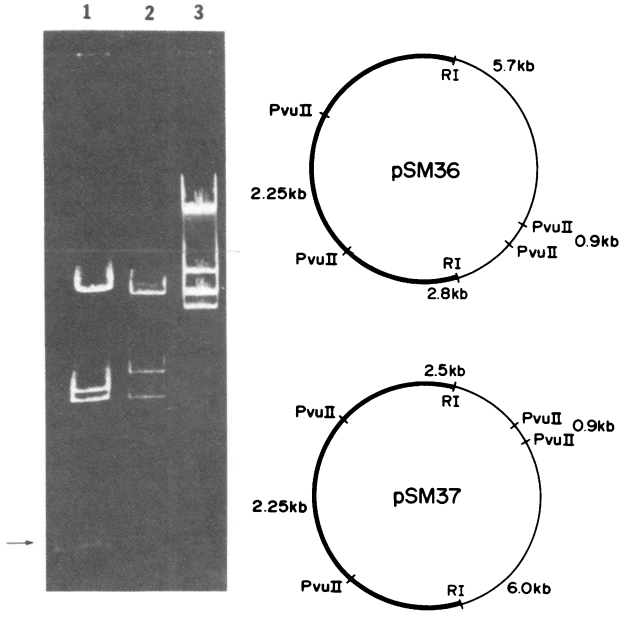

FIG. 2. (*Left*) *Pvu* II digestion patterns of pHV33 containing the 4.6-kbp insert in both orientations (lanes: 1, pSM36; 2, pSM37). The cloning vehicle, pHV33, contained two *Pvu* II restriction sites as did the 4.6-kbp insert resulting in the generation of four fragments (see *Right*). The two smaller fragments were the same size regardless of orientation; the larger of these two was pHV33 DNA, the other (arrow) was about 0.9 kbp and was contained in the 4.6-kbp insert. Lane 3: *Eco*RI-digested λ DNA fragments of (from top to bottom) 20.8 kbp, 7.1 kbp, 5.6 kbp, 5.4 kbp, 4.6 kbp, and 3.2 kbp. (*Right*) Diagram of pSM36 and pSM37 showing the opposite orientations of the fragment, the *Pvu* II cleavage sites, and the sizes of the fragments (in kbp).

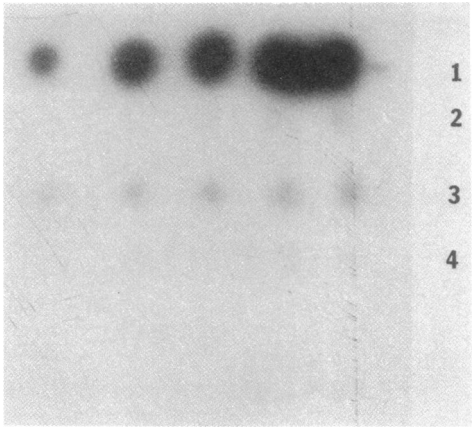

FIG. 3. Radioimmunoassay for *B. thuringiensis* protoxin in *B. subtilis* JH641 transformed with pSM36. Rows: 1, an extract from purified *B. thuringiensis* crystals containing various amounts (10–50 μg) of protein; 2, 3–15 μg of cell extract protein from *B. subtilis* JH641 containing pSM36; 3, a different extract of JH641 containing pSM36 at 4–20 μg of protein; 4, 4–20 μg of protein from extracts of JH641 containing pHV33.

Localization of the Protoxin Gene. A DNA fragment of 3.3–3.6 kbp is required to encode the structural gene for a 134,000-dalton protoxin so the *Eco*RI fragment of 4.6 kbp should consist predominantly of the protoxin gene. The plasmid pSM36 was used, therefore, as a probe to further localize the protoxin structural gene in *B. thuringiensis* chromosomal and plasmid DNA. Several independent isolates of acrystalliferous mutants lacked plasmids (4) and served as a source of chromosomal DNA. Only a single *Hin*dIII fragment of about 12 kbp present in chromosomal or plasmid digests hybridized to the pSM36 probe. Similarly, only a single *Eco*RI fragment of 4.6 kbp was found in the chromosomal and plasmid digests (unpublished results). Differences were found in a *Pvu* II digest, however, in that there were fragments of 5.6 and 0.9 kbp hybridizing in chromosomal digests but only a fragment of about 4.5 kbp in a total plasmid preparation (Figs. 4 and 5). A *Pvu* II digest of the probe, pSM36, contained a 0.9-kbp fragment as well as a larger fragment linked to the cloning vector (Fig. 2). Essentially the same pattern was found in a *Pvu* II digest of wild-type *B. thuringiensis* DNA (Fig. 4). In this case, the DNA consisted of a mixture of chromosomal and plasmid components and the fragments hybridizing to pSM36 were a composite of the two populations. There was hybridization to a 0.9-kbp fragment, indicating that its presence in the mutants was not due to an alteration related to the treatment of the cells resulting in plasmid curing. It was also apparent that the large *Pvu* II fragments derived from plasmid or chromosomal DNA differed slightly in size. The presence of the smaller *Pvu* II fragment in both the clone and the chromosomal digests indicated that a chromosomal gene had been cloned.

The major fragment of plasmid or chromosomal origin hybridizing to pSM36 in two digests was the same size and was close in *Pvu* II digests, implying that the protoxin genes were in very similar environments. A more direct comparison of the extent of similarity of the two genes was made by hybridizing under more or less restrictive conditions (19)—i.e., by omitting the low-ionic-strength wash (Fig. 5)—there was little difference

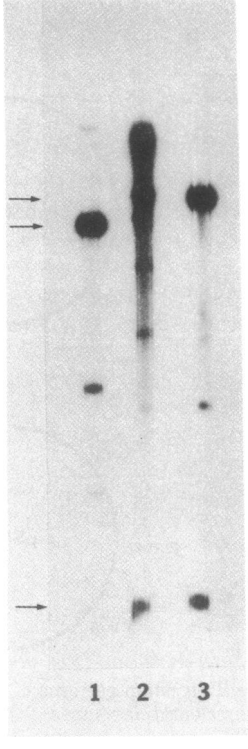

FIG. 4. Hybridization of ^{32}P-labeled pSM36 to Southern blots of *Pvu* II digests of *B. thuringiensis* plasmids (lane 1), *B. thuringiensis* total DNA (lane 2), and mutant R6 DNA (lane 3). The 0.9-kbp fragment is present in lanes 2 and 3. The other major large fragment (4.5–6 kbp) differs slightly in size in plasmid (lane 1) and chromosomal (lane 3) digests, as is evident in lane 2. Minor bands are usually barely detectable after low-ionic-strength washes (compare with Fig. 5).

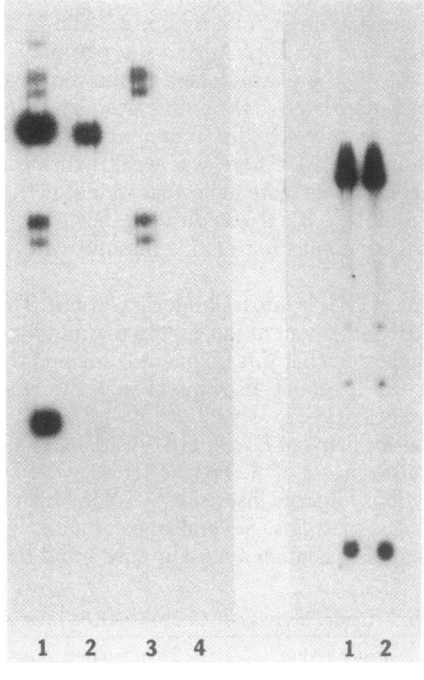

FIG. 5. Comparison between low- and high-stringency DNA·DNA hybridization of ^{32}P-labeled pSM36 DNA and *B. thuringiensis* chromosomal and plasmid DNAs. (*Left*) *Pvu* II-digested *B. thuringiensis* plasmid DNA under low-stringency conditions (lane 1). Seven fragments hybridized to DNA in contrast to one major fragment under high-stringency conditions (lane 2). Four of the fragments in lane 1, however, were attributed to the hybridization of pHV33 DNA to *B. thuringiensis* plasmid DNA (lane 3), which could be eliminated by a low-ionic-strength wash (lane 4). (*Right*) *Pvu* II-digested *B. thuringiensis* R6 DNA hybridized to pSM36 with the low-ionic-strength wash omitted (lane 1) and included (lane 2). The two major bands are about 5.0 and 0.9 kbp. The source of the minor bands is not known.

in the extent of hybridization of the major *Pvu* II fragment from plasmids (*Left*) or chromosomal DNA (*Right*). These bands were cut from the nitrocellulose and assayed in a scintillation counter. The difference in Fig. 5 *Left*, lanes 1 and 2, was about 50%, which could be due wholly or partly to the relative concentrations of DNA on the blots. There was no difference in Fig. 5 *Right*, lanes 1 and 2. Because there was no major increase in hybridization under conditions of at least 35% mismatch (19), the plasmid sequence in the large *Pvu* II fragment must be very similar or identical to that in the clone (i.e., chromosomal).

DISCUSSION

E. coli infected with a recombinant Charon 4A phage, C4K6c, produced protein that reacted with antibodies specific for the crystalline protoxin protein of *B. thuringiensis*. Alkali extracts of lysates of C4K6c-infected cells were toxic to neonate larvae of the tobacco hornworm and the toxicity data were consistent with the quantity of antigen found in lysates of phage-infected cells (about 0.1% of the total *E. coli* protein). The antigen produced in these infected cells was the same size (134,000 daltons) as the protoxin molecule obtained by alkali solubilization of *B. thuringiensis* parasporal crystals (unpublished results).

A subclone of C4K6c DNA containing a 4.6-kbp *Eco*RI fragment produced protoxin antigen in both *E. coli* and *B. subtilis* and when the cloned fragment was in either orientation. In maxicell experiments, a polypeptide of about 130,000 daltons was produced and toxicity was obtained with extracts of one subclone in pBR328, although more extensive data are needed to calculate dose–response values as in Table 1. In addition, a similar 4.6-kbp *Eco*RI fragment was found in a clone derived from a partial *Sau*3A digest of purified plasmid DNA. This clone had been isolated on the basis of production of protoxin antigen in a colony screening procedure so two independent approaches, one starting with total DNA and the other with plasmid DNA, resulted in the isolation of a protoxin gene. As discussed below, these were distinguishable in *Pvu* II digests (Figs. 2, 4, and 5).

A polypeptide of 134,000 daltons requires 3.3–3.6 kbp of coding sequences exclusive of regulatory regions. The major portion of the 4.6-kbp fragment must be encoding the protoxin gene, therefore, and was used as a probe for localizing protoxin gene regions in purified plasmids and in DNA extracted from acrystalliferous mutants. The same size *Eco*RI and *Hin*dIII fragments from the plasmid, total wild-type, and mutant DNAs hybridized to this probe. Differences were found, however, between *Pvu* II digests of the mutants and plasmid DNAs (Figs. 4 and 5). The wild type contained both sets of reacting fragments, showing the existence of the gene in two environments, a 45-kbp plasmid and the "chromosomal fraction."

The 0.9-kbp *Pvu* II fragment in the wild type and mutants was also present in a *Pvu* II digest of the clone (Fig. 2), indicating that the original Charon 4A clone contained the gene from a location other than the 45-kbp plasmid, presumably chromosomal. It is possible that plasmids of 150–200 kbp are present but were nicked and included in the chromosomal fraction, and thus they could be the location for a copy of the gene. Plasmids larger than 70 kbp were not found, although they have been reported to be present and perhaps involved in protoxin synthesis (7). In reconstruction experiments, *Agrobacterium tumefaciens* A277 was added to wild-type or mutant cells prior to lysis and the *ca.* 200-kbp Ti plasmid was recovered (unpublished results) so the procedures used were sufficiently gentle to detect plasmids of at least this size.

No protoxin antigen was detected in cell extracts of these mutants despite the presence of a gene copy that is functional when cloned from the wild type. Protoxin synthesis occurs at a defined stage of sporulation and may constitute as much as 25% of the total protein synthesis at that time (20) so special regulatory mechanisms, perhaps involving plasmid gene dosage, may be required for the synthesis of this protein.

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